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Annual Report  
Christine Lynn Lanning

Summary of Proposed Goals for the First Year:

- 1). Establish a Protein Response
- 2). Demonstrate that Chlorpyrifos Induces MDR

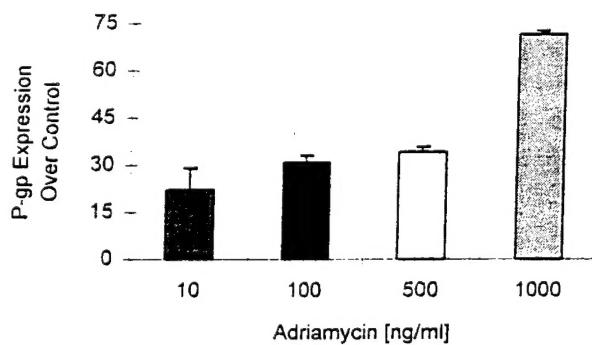
Proposed Hypothesis:

We originally proposed that chlorpyrifos, the widely used organophosphorus pesticide, induces the expression of the multidrug resistance (mdr) gene product in a manner similar to that of chemotherapeutic agents and may contribute to a MDR phenotype seen in untreated and relapsed breast cancer patients.

## RESULTS

### *A. Chlorpyrifos & P-gp Expression in MCF-7 Breast Cancer Cells*

Since treatment of rats with chlorpyrifos increased P-gp expression along the digestive tract (Lanning et al., 1995), we wanted to determine its potential to induce P-gp expression in breast cancer cells. Using flow cytometry with the C219 and UIC2 P-gp antibodies, P-gp expression in MCF-7 breast cancer cells exposed to chlorpyrifos for 3 days was examined. A significant reproducible increase in P-gp expression was not obtained with chlorpyrifos exposure. To ensure that the technique was working properly, another experiment with adriamycin, an agent known to induce P-gp expression, was conducted. A dose dependent increase in P-gp expression was observed (Figure 1). Since the technique was working properly, it was concluded that either chlorpyrifos did not induce P-gp expression or that the breast cancer cells could not metabolize chlorpyrifos to the oxon, the metabolite shown to interact with P-gp (Lanning et al., 1995).



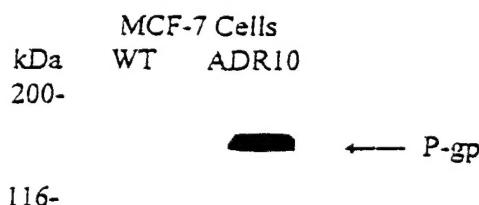
**Figure 1.** Adriamycin Induced P-gp Expression in MCF-7 Cells.

MCF-7 breast cancer cells were exposed 3 days to varying concentrations of adriamycin. P-gp expression was measured using flow cytometry with the UIC2 P-gp antibody. P-gp expression was reported as the increase over control, wild type MCF-7 cells without drug exposure.

### B. DDT Interacts with Breast Cancer P-gp

Since we were unable to establish a reproducible increase in P-gp expression with chlorpyrifos treatment, we sought a new compound. An association between elevated organochlorine levels (DDT/DDE) and the development of breast cancer has been reported (Wolff et al., 1993). We decided to examine DDT and DDE to determine any association with P-gp in breast cancer cells.

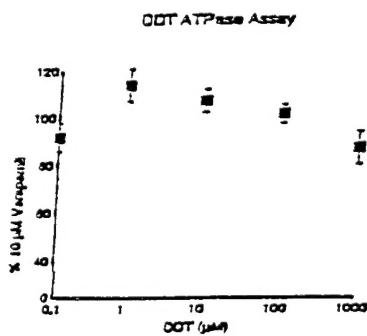
Before dosing cells with DDT to determine its impact on P-gp expression, an ATPase assay was run to determine if DDT was a substrate for P-gp. The ATPase assay measures the organic phosphate liberated from the binding of agents to P-gp. Briefly, ADR10 breast cancer cells have been selected with the P-gp agent, adriamycin, and as a result over-express P-gp (Figure 2).



**Figure 2.** P-gp Expression in ADR10 Breast Cancer Cells.

P-gp expression was determined using the P-gp antibody, C219. Lane 1, wild type MCF-7 cells, lane 2, ADR10 selected MCF-7 breast cancer cells.

Microsomes prepared from the ADR10 breast cancer cells were incubated in the presence of verapamil, an agent known to bind to P-gp and stimulate P-gp ATPase activity, and in the presence of varying concentrations of DDT. Both the control, verapamil, and DDT stimulated ATPase activity (Figure 3). DDT at 1.0  $\mu$ M stimulated maximal ATPase activity (258 nmol phosphate/min/mg protein). Interestingly, maximal stimulation with verapamil, a known agent for P-gp, (225 nmol/min/mg protein) occurred at 10  $\mu$ M. These results suggest that P-gp had a greater affinity for DDT and suggest that DDT is a substrate.

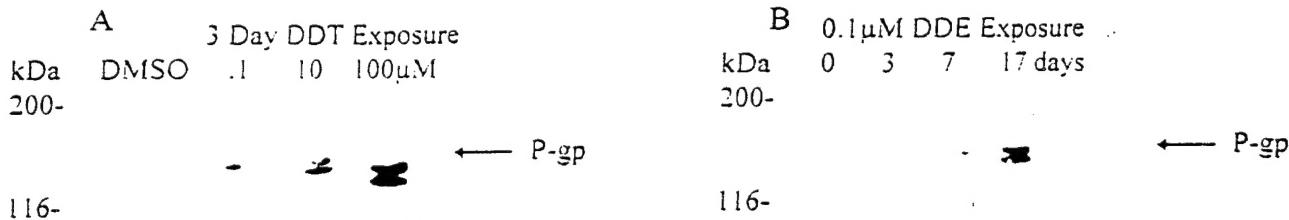


**Figure 3.** DDT Stimulation of P-gp ATPase Activity.

Membranes from ADR10 cells were incubated for 20 minutes in the presence and absence of varying concentrations of DDT. The differences between the ATPase activities measured in the presence and absence of DDT is plotted as percent of 10  $\mu$ M verapamil stimulation. Experiments were repeated at least three times.

### C. DDT and DDE Increase P-gp Expression in Breast Cancer Cells

Since DDT stimulated P-gp ATPase activity, DDT was examined to determine if it induced P-gp expression. MCF-7 breast cancer cells were treated with various concentrations of DDT and DDE for up to 17 days. P-gp expression was detected by immunoblot analysis using the P-gp antibody, C494. Increased P-gp expression was observed with both agents (Figure 4).

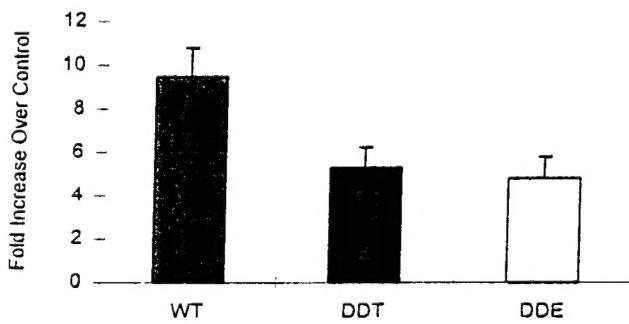


**Figure 4.** Immunoblot Analysis of DDT and DDE Treated Breast Cancer Cells.

P-gp expression was determined by immunoblot analysis of MCF-7 cells treated with various concentrations of DDT or DDE. DMSO was used as a negative control. Experiments were repeated at least three times.

### D. DDT and DDE Induce a Functional P-gp

To demonstrate that the P-gp identified in the DDT and DDE treated MCF-7 cells was functional, a Rhodamine 123 assay was performed. Briefly, Rhodamine 123 is a P-gp transported mitochondrial dye; therefore, cells with functional P-gp (ADR10, DDT, and DDE treated cells) should appear "dull" whereas cells lacking P-gp (MCF-7 wild type cells) should stain brighter. After treating wild type, ADR10, 10 μM DDT, and 10 μM DDE cells with Rhodamine 123, the staining was analyzed through flow cytometry (Figure 5). Since DDT and DDE treated MCF-7 treated cells accumulated more dye than the ADR10 cells (cell over-expressing P-gp) and less dye than the wild type MCF-7 cells (cell expressing little to no P-gp), it appeared that the P-gp was functional.



**Figure 5.** Rhodamine Accumulation in DDT and DDE Treated Breast Cancer Cells.

MCF-7 breast cancer cells were treated with 10 μM DDT or DDE for 6 days. Rhodamine accumulation was measured by flow cytometry. The results of accumulation were presented as % control (ADR10 accumulation).

## SUMMARY

Since I was unable to obtain consistent increase in P-gp expression with chlorpyrifos treatment and was able to with DDT and DDE treatment, the original hypothesis was slightly modified.

HYPOTHESIS: DDT and DDE induce the expression of the multidrug resistance (mdr) gene product in a manner similar to that of chemotherapeutic agents and this may contribute to a MDR phenotype seen in untreated and treated breast cancer patients.

The first year goal of establishing a protein response was achieved with the immunoblot analysis demonstrating that both DDT and DDE increased P-gp expression. The second goal which was scheduled to be carried over into the first 3 months of the second year was to demonstrate the induction of MDR. In so far we have demonstrated that the P-gp identified in the DDT and DDE treated cells was functional. Next we plan to demonstrate that P-gp confers resistance, the last part of this aim.

In summary, this project is in agreement with the original time schedule and we do not foresee any major changes in the time line.

## METHODS

### 1. Cell Culture

MCF-7 breast cancer cells were cultured in RPMI 1640 media supplemented with 5% heat inactivated fetal calf serum at 37°C in 5% CO<sub>2</sub>.

### 2. Flow Cytometry

P-gp expression was determined using flow cytometry by the method of Mechetner and Roninson. After washing, the MCF-7 cells were incubated with rabbit serum (1:20) for 10 minutes. After removing the serum, the primary P-gp antibody, C219 or UIC2, was applied at 5 µg/ 4x10<sup>5</sup> cells for 60 minutes at 4°C. After three washes with a 1% BSA/PBS solution, the cells were incubated for 60 minutes at 4°C with the secondary antibody, FITC conjugated rabbit antimouse IgG (1:32). The cells were washed three times and resuspended in 1 ml PBS. The fluorescence was measured by Dr. Michael Cook using a Coulter Epics 753 flow cytometer located in the Cancer Center Flow Cytometry Laboratory.

### 3. Membrane Preparation

Breast cancer cells were homogenized in a hypotonic lysis buffer (10 mM Tris, 10 mM NaCl, 2 mM EDTA, 2 mM PMSF, 10 mg/ml leupeptin, and 8 mg/ml aprotinin) and centrifuged at 400 x g for 10 minutes. The resulting supernatant was centrifuged at 100,000 x g for 60 minutes and the membrane pellet was resuspended in sample buffer and stored at -70°C.

### 4. Immunoblot Analysis

After determining the protein concentration using the BCA method, 100 µg membrane protein were resolved on a 7.5% polyacrylamide gel by the method of Laemmli. The gel was transferred for one hour to PVDF membrane and incubated for 1-2 hours in blocking solution containing 2.5% Carnation nonfat dry milk. The P-gp antibody, C219 or C494, was applied at 1 µg/ml for 1-2 hours. After washing, the membrane was incubated for 1 hour with biotinylated goat antimouse IgG diluted 1:600 in TBS containing 0.1% Tween 20. After washing, the membrane was incubated for one hour with streptavidin horseradish peroxidase diluted 1:6000 in PBS. After washing, the membrane was exposed to the ECL reagents and placed under film. Densitometry was used to quantitate the films.

### 5. P-gp ATPase Activity

The ATP hydrolysis assay was performed by the method of Sarkadi. Briefly, 10 µg ADR10 membrane proteins were incubated at 37°C in 0.1 ml of buffer [50 mM Tris-Mes (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM KCL, and 5 mM sodium azide] with various concentrations of DDT. The ATPase reaction was initiated with the addition of 5 mM ATP and terminated with the addition of 0.1 ml 5% SDS solution. The SDS-containing samples were supplemented with 0.4 ml reagent A (2.5 M H<sub>2</sub>SO<sub>4</sub>, 1% ammonium molybdate, and 0.014% antimony potassium tartrate) and 1 ml distilled water.

Finally, 0.2 ml 1% ascorbic acid was added and the optical density was measured at 880 nm. ATPase activity was estimated by the difference obtained in  $P_i$  levels between 0 and 20 minute incubation periods.

#### 6. Rhodamine 123 Accumulation Assay

Rhodamine accumulation was measured by flow cytometry following the method of Chaudhary and Roninson. Briefly,  $2 \times 10^5$  cells were incubated with 100 ng/ml Rhodamine 123 for 2 hours at 37°C. After incubating, the cells were washed with PBS and analyzed by flow cytometry.

## REFERENCES

Chaudhary PM, Roninson IB (1993). Activation of MDR1 (P-glycoprotein) gene expression in human cells by protein kinase C agonists. *Oncology Research* 7, 281-290.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 277, 680-685.

Lanning, C.L., Fine, R.L., Sachs, C.W., Rao, U.S., Corcoran, J.J., and Abou-Donia, M.B. (1995) Chlorpyrifos oxon interacts with the mammalian multidrug resistance protein, P-glycoprotein. *J. Toxicology and Environmental Health* (in press).

Mechetner, E.B., and Roninson, I.B. (1992) Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody. *Proc. Natl. Acad. Sci. USA* 89, 5824-5828.

Sarkadi, B., Price, E. M., Boucher, R. C., Germann, U. A., and Scarborough, G. A. (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J. Biol. Chem.* 267, 4854-4858.

Wiechelman, K., Brasun, R., and Fitzpatrick, J. (1988) Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *J. Anal. Biochem.* 175, 231-237.

Wolff, M.S., Toniolo, P.G., Lee, E.W., Rivera, M., and Dubin, N. (1993) Blood levels of organochlorine residues and risk of breast cancer. *J. Natl. Cancer Inst.* 85, 648-652.